Analysis of Protein Glycosylation using HILIC

Dr Morten Thaysen-Andersen, Department of Biochemistry and Molecular Biology, University of Southern Denmark

Introduction

The attachment of monosaccharides to proteins, known as protein glycosylation, is an abundant post-translational modification (PTM). It is widely accepted that protein glycosylation is involved in numerous essential cellular processes. Thus, the structural and functional characterizations of these PTMs are important and functional glycomics and glycoproteomics are rapidly growing research areas. One of the most prominent challenges associated with these disciplines is the substoichiometric presence of the glycosylation, resulting from substantial heterogeneity of the attached carbohydrates (collectively called glycans) as well as only partial glycan occupancy of a given site. Fractionation and enrichment of the glycosylated species are consequently essential to alleviate this problem. However, the significant hydrophilicities associated with these biomolecules, which arise from the numerous hydroxyl groups of the glycan, limit the use of traditional chromatographic techniques such as reversed-phase liquid chromatography (LC) setups. In contrast, HILIC is an attractive chromatographic technique for the analysis of the glycoconjugates and applications usually involve detection by mass spectrometry (MS) to benefit from its high sensitivity, accuracy and resolution as well as its high throughput potential. Several HILIC stationary phases with different functional groups are available and solvents with high content of organic component (40-97%) in water are typically used for the mobile phase[1]. Since it can be difficult to dissolve glycans and glycopeptides, if the amount of organic is very high, 80% organic content is often used in the mobile phase as starting condition. Acetonitrile is by far the most popular organic modifier and to hydrate the stationary phase and generate reproducible results, at least 3% water should be present. Suitable buffers include ammonium salts of acetate and formate due to their volatibility and excellent solubility in organic solvent. These salts have been shown to minimize the electrostatic interactions (repulsions or attractions) between charged stationary phases and analytes i.e. sialic acids containing glycoconjugates[2]. Formate and other weak acids are often-used mobile phase additives for adjusting pH when using on-line HILIC-MS setups.

Various HILIC applications for the analysis of released (free) glycans and glycopeptides are summarized in the following. The applications range from sample preparations using HILIC in solid-phase extraction (SPE) formats to chromatographic separation using analytical and capillary-scale HILIC columns with both off- and on-line MS detection. An overview of the typical workflows in glycomics and glycoproteomics is given in Figure 1 (see page 4).

Sample preparation of glycans and glycopeptides using HILIC SPE

Among various other enrichment techniques e.g. lectins, graphitized carbon and hydrazide chemistry, HILIC SPE has been used increasingly in the sample preparation of glycoconjugates. Applications vary from enrichment of glycopeptides from simple and complex peptide mixtures to desalting of glycans and glycopeptides.

HILIC-based sample preparation has been performed utilizing a variety of stationary/mobile phases and SPE formats. When choosing SPE format it is important to consider the column/resin capacity and sample volume/loading speed. Solvents used for HILIC SPE purifications are usually similar to the ones used in regular HILIC separation, but vary slightly depending on the specific experiment and the down-stream detection methods. Following column equilibration, load and wash in a suitable mobile phase of high organic content, the elution in HILIC SPE is normally performed as a one-step elution by switching to an aqueous mobile phase either without or with a low concentration of organic solvent (typically 0-30% acetonitrile). Thus, water is the stronger eluting solvent, and a small amount of weak acid (e.g. 0.5-2% formic acid) is often included to generate protonated species for MS. The one-step elution is carried out since fractionation of the retained compounds often is not desired. The use of MS-friendly solvents allows subsequent analysis of the enriched glycopeptide/glycan fractions by electrospray ionization MS (ESI-MS) or matrix-assisted laser desorption/ionization MS (MALDI-MS).

Enrichment of glycopeptides from simple peptide mixtures using HILIC SPE

The combination of a relative poor ionization efficiency of glycopeptides with respect to the unmodified peptides and the 'dilution' of signals resulting from the distribution of multiple glycoforms at the glycosylation sites, call for efficient enrichment of glycopeptides from the sample prior to MS detection. The rationale is that the hydrophilic contribution from the glycan often is sufficient to generate a rather unique overall hydrophilicity among the glycopeptides, and this physicochemical property can be used to separate these from the less hydrophilic non-glycosylated peptides on polar stationary phases using hydrophilic interactions. As the hydrophilic contribution increases with glycan size (and charge), applications have mainly been presented for N-linked glycopeptides, since N-glycans tend to be larger than glycans of the O-linked type. Typically, it is preferred that the non-glycosylated peptides appear in the flow-through, while the glycopeptides are selectively retained on the column and can be eluted separately. As salts and detergents are found in the flow-through/wash fractions, desalting of the sample is simultaneously performed. Desalting is important, as salts interfere with the MS detection if present.

One very useful HILIC SPE application is the enrichment of glycopeptides from relatively simple mixtures e.g. tryptic digests of purified or semi-purified glycoproteins. Here, HILIC has been shown to be capable of selectively retaining the glycopeptides. The resulting depletion of non-glycosylated peptides is essential when MALDI-MS or infusion ESI-MS is used for downstream detection, where no further analyte separation is performed prior to MS detection. The efficiency of HILIC SPE for purifying glycopeptides has been shown for a number of glycoproteins and with a variety of stationary/mobile phases and formats (please consult references for further details on the individual experiments)[3-9]. It is important to stress that if a peptide mixture is analyzed directly without any purification or with desalting using the conventional hydrophobic resin (Poros R2), the signals for the glycopeptides are usually undetectable due to signal dilution and suppression effects from non-glycosylated peptides. This demonstrates the importance of enriching for glycopeptides.

Enrichment of glycopeptides from complex mixture using HILIC SPE

HILIC SPE has also been used to enrich glycopeptides from complex peptide mixtures such as proteolytic digests of serum, saliva and other body fluids. This approach has mostly been used to determine N-glycosylation sites of glycoproteins following glycan release[10-12]. The glycan release leaves a peptide mass increment of 1 Da or 203/349 Da if N-glycosidase F or endo-β-N-acetylglucosaminidase is used for deglycosylation, respectively. Hence, traditional proteomics workflows can be used for such types of experiments and data interpretation can be performed using existing search engines. Alternatively, the approach can be used to analyze the native glycopeptides, with characterization of both the peptide and the glycan moieties. However, this has only rarely been performed due to the lack of adequate bioinformatic tools and search engines making data interpretation difficult and time-consuming. It is expected that the development of such bioinformatic tools will promote much more focus in this direction.

Even if HILIC SPE is capable of making a complete separation of glycopeptides and non-glycosylated peptides, the sample can still be rather complex since a large fraction of the proteome is glycosylated. Hence, an additional level of separation is still required in front of the mass spectrometer. For that reason reversed-phase LC-ESI-MS is an attractive technique to use in combination with HILIC SPE when analyzing samples of high complexity.

Merck SeQuant AB · Box 7956 · S-907 19 Umeå · Sweden
Tel: +46-90-154880 · Fax: +46-90-154883
info@mercksequant.com · www.mercksequant.com

Improved enrichment of glycopeptides using ion-pairing HILIC SPE

Although HILIC SPE has proved rather efficient (reproducible, sensitive and specific) for the enrichment of glycopeptides from mixtures, hydrophilic non-glycosylated peptides are regularly observed to be co-enriched with the glycopeptides, in particular from complex peptide mixtures. This co-enrichment, which arises from a hydrophilic overlap between glycopeptides and non-glycosylated peptides, often results in suppression of the glycopeptide ionization during MS analysis. It was recently reported that the addition of an ion-pairing reagent such as trifluoroacetic acid or HCl can act to decrease this overlap[13,14]. This was demonstrated by the improved separation of glycopeptides and non-glycosylated peptides using on-line ion-pairing HILIC ESI MS. Supposedly, ion-pairing affects the overall hydrophilicity of non-glycosylated peptides to a greater extent than the glycopeptides (hydroxyl groups of the glycan are not affected), separating the two groups in terms of hydrophilicity. A similar increase in the glycopeptide enrichment efficiency has lately been demonstrated for ion-pairing HILIC SPE[15]. The improved enrichment efficiency was demonstrated for a wide range of sample complexities i.e. from digests of purified glycoproteins to crude plasma samples. In addition, it was shown that ion-pairing is beneficial for glycopeptide enrichment from multiple stationary phases.

Desalting of glycans and glycopeptides using HILIC SPE

For traditional glycomics experiments, where the aim is to determine the global glycan profile, HILIC SPE also represents an ideal technique for clean-up (desalting) of released glycans prior to MS. The release of N- and O-linked glycans from the total pool of glycoproteins are commonly performed using enzymatic or chemical release. Due to the lower mass and the hydrophilic nature, the released glycans are easily purified from the proteins. Following isolation using high molecular mass cut-off filters/membranes, the glycans are often derivatized using a variety of methods e.g. fluorescence labeling[16] or permethylation[17] in order to enhance detection and identification of the glycans. The introduction of salts and detergents in these derivatization steps calls for thorough desalting of the sample prior to LC and/or MS detection. Together with graphitized carbon, HILIC SPE represents a widely used method for glycan clean-up and, as described below, desalting of glycans (and glycopeptides) can be performed without loss of quantitative information.

Although HILIC is considered to be a non-biased matrix that do not select for certain structural classes or subclasses of the glycome/glycoproteome, all sample handling, including purification, can potentially generate a bias by changing the composition of the species in a given mixture with a resulting loss of quantitative information. In a recent study, it was shown that HILIC essentially is non-biased in the desalting of glycans and glycopeptides when working below the capacity limit of the HILIC SPE column. However, biases were introduced, when the column capacities were exceeded. This was a result of competitive binding where the least hydrophilic glycopeptides/glycans were outcompeted by the more hydrophilic glycopeptides/glycans. This trend was observed for a variety of HILIC stationary phases and stresses the need for ensuring sufficient column capacity when performing HILIC SPE purification. For glycopeptide enrichment from complex mixtures, however, it should be noted that columns with capacities far greater than needed may increase the binding of non-glycosylated peptides (Thaysen-Andersen, unpublished data). Thus, the column capacity should be optimized to the analyte amount and the sample complexity.

HILIC separation of glycans and glycopeptides

From a chromatographic viewpoint, HILIC is an attractive technique for retention and separation of polar compounds such as glycans and glycopeptides. Applications include separations of different glycoforms of already purified glycan/glycopeptide fractions with off- or on-line MS detection as well as more crude separations of non-glycosylated species from glycoconjugates in pre-fractionation approaches. The retention mechanism of HILIC varies depending on the functional group of the stationary phase and the analyte, as well as the nature of the mobile phase. Thus, the stationary and mobile phases are essential parameters to consider for optimal analyte retention and separation.

HILIC separation of glycans with off-line and on-line MS detection

Separation and profiling of released glycans using HILIC (alternatively called normal-phase HPLC when used for this purpose in some parts of the literature) coupled with a fluorescence detector has been one of the standard techniques for decades[18]. Often analytical-scale HILIC columns are used and detection limits around 50-100 femtomol for 2-AB labeled glycans have been reported[19]. In a typical setup, a mixture of high acetonitrile amount and 50 mM ammonia formate in water, pH 4.4, is used as the eluent. Gradient elution is normally performed by slowly decreasing the concentration of the organic solvent. This approach benefits from easy quantitation of the separated glycans by measuring the peak area of the eluting analytes. Another advantage is that not only the size but also the structure of the glycans affect the retention time often providing information of isomeric structures, which can not be distinguished in MS due to identical m/z. However, the technique is limited by poor resolution and through-put compared to MS detection. Identification of the eluting compounds is usually performed by matching retention times to an obtained library and/or to a reference dextran ladder or simply by collection of the eluted fractions with subsequent MS detection.

The use of high polar organic/low aqueous solvent content in the HILIC mobile phase is ideal for direct ESI-MS detection, and this on-line approach has been used increasingly in recent years[20]. Analysis of released glycans have been achieved with great success using on-line HILIC-ESI-MS with various stationary phases, glycan types (e.g. N- and O-linked) and glycan derivatization levels (e.g. underivatized, 2-aminobenzamide and 8-amino-naphthalene-1,3,6-trisulfonate labeled glycans)[21-23]. Regular HILIC conditions are used for on-line approaches: high concentration of organic solvent and low concentration of volatile salts and weak acids (i.e. formic acid or acetic acid). It is important, that the amount of ion pairing agents like trifluoroacetic acid is minimized, because it reduces the MS sensitivity significantly. The elution of glycans is normally observed above 50% acetonitrile. The detection is typically performed in positive ionization mode; however, negative ionization mode has also proved efficient in particular for sialylated and sulfated glycans[2]. Most studies using on-line HILIC-ESI-MS use flow rates of 40-200 µl/min, but nanoflows (300 nl/min) have also been reported, where detection limits in the low femtomol range have been achieved[21,23]. Lately, a chip-based HILIC-ESI-MS platform has been introduced, where glycosaminoglycans are separated and profiled in negative ionization mode[24].

HILIC separation of glycopeptides with off-line and on-line MS detection

Although not widely used, glycopeptides can be separated by HILIC and detected by their UV absorbance. Since the glycans have almost no UV absorbance, the detection is based on the absorbance of the peptide moiety. The eluted fractions are then collected and characterized using MS in an off-line setup. This approach has been used to quantitatively monitor variations in the microheterogeneity of glycoproteins[25]. Off-line HILIC-MS can also be used for pre-fractionation of glycopeptides from non-glycosylated peptides originating from crude mixtures, since glycopeptides in general will be more hydrophilic and therefore better retained on HILIC. However, due to the likely presence of hydrophilic non-glycosylated peptides in the glycopeptide fractions, a secondary separation step is required prior to detection (e.g. on-line reversed-phase-LC-MS).

The last few years there have been some reports of glycopeptide separation using HILIC with on-line MS detection, and as the field of functional glycomics and glycoproteomics are moving towards site-specific approaches, glycopeptide profiling is expected to be used increasingly in the future. It has been shown that N- and O-linked glycopeptides of specific proteins can be profiled using analytical-scale[2,26] and nano-scale[27-29] HILIC columns with on-line MS detection. Characterization is performed by glycopeptide fragmentation in tandem MS, which is optimally carried out by a combination of collision-induced dissociation and electron transfer dissociation to obtain information of both the glycan and the peptide moieties.

Merck SeQuant AB · Box 7956 · S-907 19 Umeå · Sweden
Tel: +46-90-154880 · Fax: +46-90-154883
info@mercksequant.com · www.mercksequant.com

Conclusion

The proteomic field has a strong focus on PTM characterization, in particular protein phosphorylation and glycosylation. This arises from the strong involvement of these modifications in the modulation of protein functions. Therefore, a considerable effort has been put into expanding the classical proteomics strategies to enable PTM mapping, so called modification-specific proteomics. The complementary nature of HILIC compared to standard chromatographic techniques and its MS compatibility makes HILIC a popular technique in proteomics. HILIC is especially suitable for separation and enrichment of hydrophilic PTMs such as glycosylation. The ability to retain peptides containing these hydrophilic modifications is a very attractive feature of HILIC and it has greatly expanded the set of biomolecules that can be analyzed using MS. However, it seems that the full potential of HILIC is not yet exploited and significant improvements must still be obtained before glycans and glycopeptides routinely will be analyzed by HILIC-MS. In particular, it is important to establish the retention mechanisms of the glycoconjugates on the different HILIC phases and the influence of various solvents and additives.

It is evident from the data already available that HILIC is a worthy complement to reversed-phase chromatography. Thus, it is expected that HILIC gradually will move into the proteomic field as a new and complementary technique in the separation tool-box that will be used primarily for the analysis of hydrophilic modifications like protein glycosylation, but also for unmodified hydrophilic peptides that with the existing techniques are difficult to analyze.

References

1. Hemstrom, P. & Irgum, K. Hydrophilic interaction chromatography. J Sep Sci 29, 1784-821 (2006).
2. Takegawa, Y. et al. Simple separation of isomeric sialylated N-glycopeptides by a zwitterionic type of hydrophilic interaction chromatography. Journal of Separation Science 29, 2533-2540 (2006).
3. Thaysen-Andersen, M. et al. Rapid and individual-specific glycoprofiling of the low abundance N-glycosylated protein tissue inhibitor of metalloproteinases-1. Mol Cell Proteomics 6, 638-47 (2007).
4. Thaysen-Andersen, M. et al. Investigating the biomarker potential of glycoproteins using comparative glycoprofiling - application to tissue inhibitor of metalloproteinases-1. Biochim Biophys Acta 1784, 455-63 (2008).
5. Omaetxebarria, M.J. et al. Isolation and characterization of glycosylphosphatidylinositol-anchored peptides by hydrophilic interaction chromatography and MALDI tandem mass spectrometry. Analytical Chemistry 78, 3335-3341 (2006).
6. Tajiri, M., Yoshida, S. & Wada, Y. Differential analysis of site-specific glycans on plasma and cellular fibronectins: application of a hydrophilic affinity method for glycopeptide enrichment. Glycobiology 15, 1332-1340 (2005).
7. Wada, Y., Tajiri, M. & Yoshida, S. Hydrophilic affinity isolation and MALDI multiple-stage tandem mass spectrometry of glycopeptides for glycoproteomics. Analytical Chemistry 76, 6560-6565 (2004).
8. Yu, Y.Q., Fournier, J., Gilar, M. & Gebler, J.C. Identification of N-linked glycosylation sites using glycoprotein digestion with pronase prior to MALDI tandem time-of-flight mass spectrometry. Analytical Chemistry 79, 1731-1738 (2007).
9. Yu, Y.Q., Gilar, M., Kaska, J. & Gebler, J.C. A rapid sample preparation method for mass spectrometric characterization of N-linked glycans. Rapid Communications in Mass Spectrometry 19, 2331-2336 (2005).
10. Hagglund, P., Bunkenborg, J., Elortza, F., Jensen, O.N. & Roepstorff, P. A new strategy for identification of N-glycosylated proteins and unambiguous assignment of their glycosylation sites using HILIC enrichment and partial deglycosylation. Journal of Proteome Research 3, 556-566 (2004).
11. Hagglund, P. et al. An enzymatic deglycosylation scheme enabling identification of core fucosylated N-glycans and O-glycosylation site mapping of human plasma proteins. J Proteome Res 6, 3021-31 (2007).
12. Picariello, G., Ferranti, P., Mamone, G., Roepstorff, P. & Addeo, F. Identification of N-linked glycoproteins in human milk by hydrophilic interaction liquid chromatography and mass spectrometry. Proteomics 8, 3833-3847 (2008).
13. Ding, W., Hill, J.J. & Kelly, J. Selective enrichment of glycopeptides from glycoprotein digests using ion-pairing normal-phase liquid chromatography. Anal Chem 79, 8891-9 (2007).
14. Ding, W., Nothaft, H., Szymanski, C.M. & Kelly, J. Identification and quantification of glycoproteins using ion-pairing normal-phase liquid chromatography and mass spectrometry. Mol Cell Proteomics 8, 2170-85 (2009).
15. Mysling, S., Palmisano, G., Højrup, P. & Thaysen-Andersen, M. Utilizing Ion-Pairing Hydrophilic Interaction Chromatography Solid Phase Extraction for Efficient Glycopeptide Enrichment in Glycoproteomics. Submitted paper (2010).
16. Anumula, K.R. & Dhume, S.T. High resolution and high sensitivity methods for oligosaccharide mapping and characterization by normal phase high performance liquid chromatography following derivatization with highly fluorescent anthranilic acid. Glycobiology 8, 685-94 (1998).
17. Ciucanu, I. & Costello, C.E. Elimination of oxidative degradation during the per-O-methylation of carbohydrates. J Am Chem Soc 125, 16213-9 (2003).
18. Anumula, K.R. Advances in fluorescence derivatization methods for high-performance liquid chromatographic analysis of glycoprotein carbohydrates. Anal Biochem 350, 1-23 (2006).
19. Rudd, P.M. et al. A high-performance liquid chromatography based strategy for rapid, sensitive sequencing of N-linked oligosaccharide modifications to proteins in sodium dodecyl sulphate polyacrylamide electrophoresis gel bands. Proteomics 1, 285-94 (2001).
20. Nguyen, H.P. & Schug, K.A. The advantages of ESI-MS detection in conjunction with HILIC mode separations: Fundamentals and applications. J Sep Sci 31, 1465-80 (2008).
21. Wuhrer, M., Koeleman, C.A., Deelder, A.M. & Hokke, C.H. Normal-phase nanoscale liquid chromatography-mass spectrometry of underivatized oligosaccharides at low-femtomole sensitivity. Anal Chem 76, 833-8 (2004).
22. Charlwood, J., Birrell, H., Bouvier, E.S., Langridge, J. & Camilleri, P. Analysis of oligosaccharides by microbore high-performance liquid chromatography. Anal Chem 72, 1469-74 (2000).
23. Wuhrer, M., Koeleman, C.A.M., Hokke, C.H. & Deelder, A.M. Nano-scale liquid chromatography-mass spectrometry of 2-aminobenzamide-labeled oligosaccharides at low femtomole sensitivity. International Journal of Mass Spectrometry 232, 51-57 (2004).
24. Staples, G.O. et al. A chip-based amide-HILIC LC/MS platform for glycosaminoglycan glycomics profiling. Proteomics 9, 686-95 (2009).
25. Zhang, J. & Wang, D.I. Quantitative analysis and process monitoring of site-specific glycosylation microheterogeneity in recombinant human interferon-gamma from Chinese hamster ovary cell culture by hydrophilic interaction chromatography. J Chromatogr B Biomed Sci Appl 712, 73-82 (1998).
26. Takegawa, Y. et al. Separation of isomeric 2-aminopyridine derivatized N-glycans and N-glycopeptides of human serum immunoglobulin G by using a zwitterionic type of hydrophilic-interaction chromatography. J Chromatogr A 1113, 177-81 (2006).
27. Wuhrer, M., Koeleman, C.A., Hokke, C.H. & Deelder, A.M. Protein glycosylation analyzed by normal-phase nano-liquid chromatography--mass spectrometry of glycopeptides. Anal Chem 77, 886-94 (2005).
28. Takegawa, Y. et al. Profiling of N- and O-glycopeptides of erythropoietin by capillary zwitterionic type of hydrophilic interaction chromatography/electrospray ionization mass spectrometry. J Sep Sci 31, 1585-93 (2008).
29. Wuhrer, M., de Boer, A.R. & Deelder, A.M. Structural glycomics using hydrophilic interaction chromatography (HILIC) with mass spectrometry. Mass Spectrom Rev 28, 192-206 (2009).

About ZIC®-HILIC Chromatography

The ZIC®-HILIC stationary phase is based on the covalently bonded zwitterionic sulfobetaine group indicated in Figure 2. It is available with a silica support in 3.5, 5 and 10 µm particle sizes in various column dimensions from capillary to semi-preparative (75 µm up to 20 mm ID, It is also available on a polymeric support on 5 µm particles (ZIC®-*p*HILIC). For additional information, request a copy of the tutorial booklet A Practical Guide to HILIC, which is published by Merck SeQuant and is available free of charge.

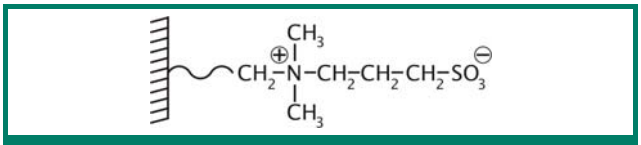

Figure 2: The Zwitterionic Sulfobetaine Group of ZIC®-HILIC.

Merck SeQuant AB · Box 7956 · S-907 19 Umeå · Sweden
Tel: +46-90-154880 · Fax: +46-90-154883
info@mercksequant.com · www.mercksequant.com

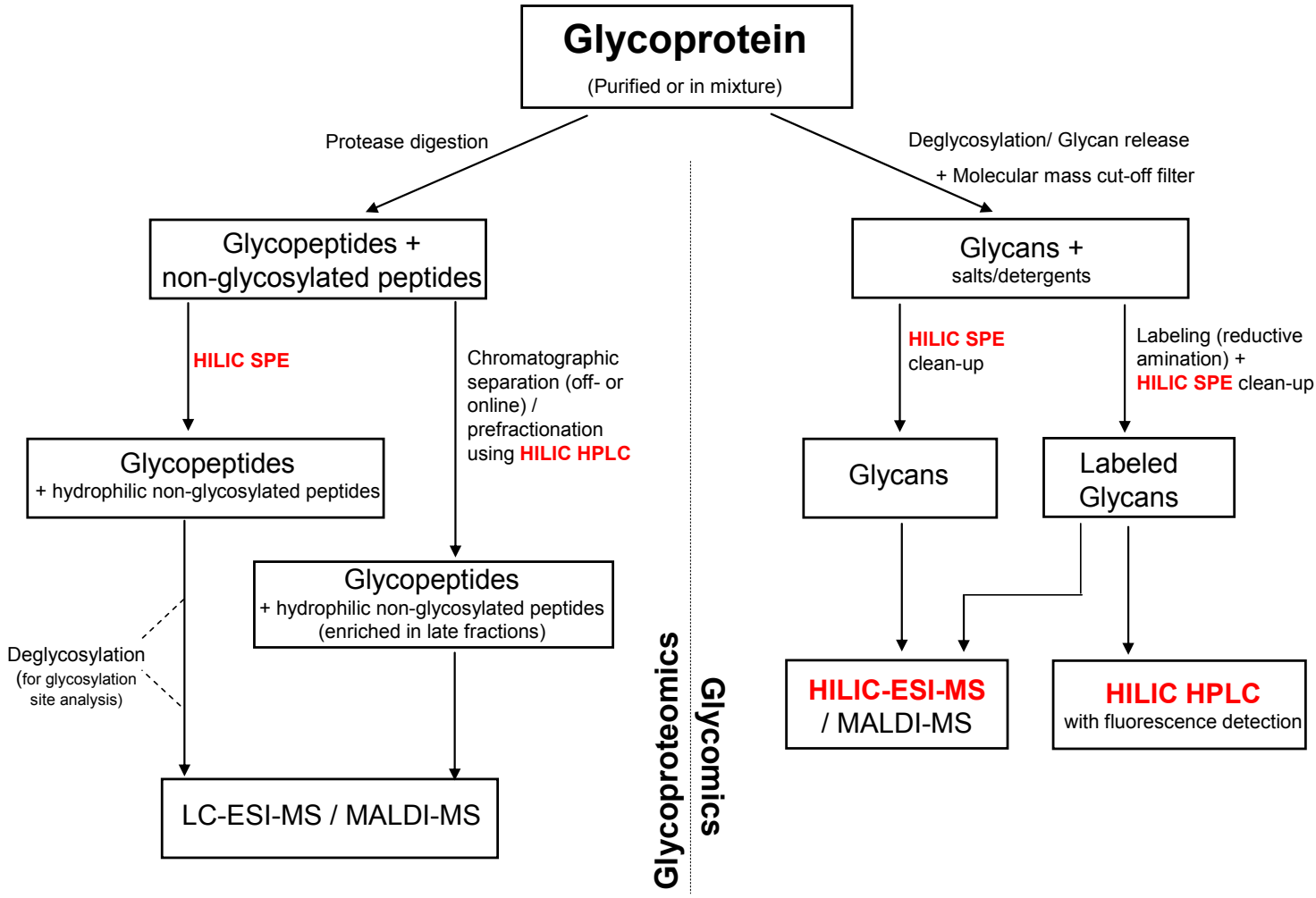

Figure 1: The flow scheme outlines the typical workflows in glycoproteomics and glycomics. The step involving HILIC is highlighted in red.

Merck SeQuant AB · Box 7956 · S-907 19 Umeå · Sweden
Tel: +46-90-154880 · Fax: +46-90-154883
info@mercksequant.com · www.mercksequant.com